Biochem. J. (1992) 281, 577–592 (Printed in Great Britain)

REVIEW ARTICLE

Phenobarbital induction of cytochrome *P*-450 gene expression

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INTRODUCTION

It has long been known that phenobarbital (PB) and numerous other drugs and chemicals induce the expression of drug- and steroid-metabolizing enzymes in liver tissue (Conney, 1967). These inductive responses can have a major impact on drug metabolism, pharmacokinetics, and drug–drug interactions, on the toxicity and carcinogenicity of foreign chemicals, and on the potency and disposition of circulating hormones (Barry & Feely, 1990; Conney, 1982; Jakoby, 1980). One of the major drug- and steroid-metabolizing enzymes that is responsive to PB induction is cytochrome *P*-450 (Fig. 1), a collection of structurally related haemoprotein mono-oxygenase enzymes that hydroxylate a large number of steroid hormones, fatty acids, drugs, carcinogens, and environmental chemicals (Ortiz de Montellano, 1986; Guengerich, 1987; Schuster, 1989; Porter & Coon, 1991). The expression in liver of individual *P*-450 enzymes (*P*-450 forms) is regulated both by endogenous factors, including gonadal and pituitary hormones (Waxman, 1988), and by drugs and other foreign compounds, many of which can dramatically induce expression of individual cytochromes *P*-450 (Nebert & Gonzalez, 1987; Ryan & Levin, 1990). Although much is known about the molecular mechanisms by which 3-methylcholanthrene and related polycyclic aromatic hydrocarbons induce liver expression of select *P*-450 genes (for reviews see Adesnik & Atchison, 1985; Whitlock, 1986, 1990; Gonzalez, 1988; Okey, 1990; Nebert *et al.*, 1990), the mechanism(s) by which PB and other chemicals of the PB inducer class increase expression of the PB-inducible *P*-450s are still poorly understood. This article reviews our current understanding of the biochemistry and molecular biology of PB-inducible *P*-450 gene expression. Recent improvements in primary hepatocyte culture techniques that should facilitate a cellular and molecular dissection of the underlying mechanisms of PB induction are also discussed. Finally, several models and working hypotheses for the mechanism of action of PB on *P*-450 gene expression are proposed and evaluated in the context of possible future research directions in this field.

PB-INDUCIBLE RAT *P*-450s 2B1 AND 2B2 AND THEIR REGULATION

Background

The cytochrome *P*-450 superfamily is comprised of at least 27 distinct gene families, 10 of which are found in mammals (Nebert *et al.*, 1991; Gonzalez, 1990). *P*-450 proteins that exhibit at least ~ 40% amino acid sequence identity are classified within the same gene family (families identified by Arabic numerals: 1, 2, 3, etc.), while *P*-450s that are > 55–60% similar are grouped within the same subfamily (subfamilies identified by capital letters: A, B, C, etc.). Individual *P*-450 forms within a subfamily are numbered sequentially, e.g. *P*-450s 2B1, 2B2, 2B3, etc. Some of the *P*-450 cytochromes are highly inducible; for example, expression of *P*-450 1A1 can be elevated 100-fold or more in liver and many extrahepatic tissues following exposure to 2,3,7,8,-tetrachlorodibenzo-*p*-dioxin (dioxin), 3-methylcholanthrene or other polycyclic aromatics (Whitlock, 1990; Pasco *et al.*, 1988). *P*-450 2E1 expression in liver is increased following exposure to ethanol, acetone, isoniazid, imidazole and other drugs and chemicals (Koop *et al.*, 1985; Thomas *et al.*, 1987), while clofibrate and other peroxisome proliferators induce the expression of the subfamily 4A *P*-450s in liver and kidney (Kimura *et al.*, 1989; Milton *et al.*, 1990; Sundseth & Waxman, 1992). Effective inducers of *P*-450 subfamily 3A include macrolide antibiotics, synthetic steroids (e.g., dexamethasone) and PB (Schuetz *et al.*, 1986; Gonzalez *et al.*, 1986), amongst others. PB, as well as a large number of structurally unrelated chemicals termed 'PB-like' inducers, can also elevate expression of individual members of the *P*-450 2A, 2B, and 2C subfamilies, both in laboratory animals (e.g., Leighton & Kemper, 1984; Waxman *et al.*, 1985; Ryan & Levin, 1990), and in humans (e.g. PB induction of 2C *P*-450s demonstrated in cultured human hepatocytes; Morel *et al.*, 1990). PB-like inducers include isosafrole, *trans*-stilbene oxide, allylisopropylacetamide, chlordane and other organochlorine pesticides, various phenothiazines, nonplanar halogenated biphenyls, and carcinogens such as acetylaminofluorene (e.g., Thomas *et al.*, 1981; Dannan *et al.*, 1983) (Fig. 2). When comparing PB to related barbiturates, the induction potency is directly correlated with the plasma half-life of the barbiturate; compounds with low rates of metabolism and long half-lives correspond to the more potent inducing agents (Ioannides & Parke, 1975). In the case of PB, the parent compound, and not its major hydroxylated metabolite, *p*-hydroxy-PB, appears to be the active inducing agent (Cresteil *et al.*, 1980).

Protein levels of some *P*-450s increase modestly following PB treatment (e.g. 2–4-fold increase of *P*-450 2C6), while others are increased dramatically in response to drug exposure (up to 50–100-fold-fold or greater increase of *P*-450 2B1) (Table 1). There is no necessary correlation between a *P*-450's gene family or subfamily classification and its responsiveness to PB (e.g. *P*-450 2B3 is not induced by PB, whereas *P*-450s 2B1 and 2B2 are PB-inducible; Labbe *et al.*, 1988). Indeed, PB induction is not limited to cytochrome *P*-450s, insofar as several other enzymes that contribute to foreign compound metabolism, including aldehyde dehydrogenase, epoxide hydrolase, NADPH: *P*-450 reductase, UDP-glucuronyl transferase and several glutathione *S*-transferases are also inducible by PB (e.g. Dunn *et al.*, 1989; Pickett *et al.*, 1981; Kuriyama *et al.*, 1969; Mackenzie *et al.*, 1984; Pickett & Lu, 1989) (Table 2). The effects of PB, unlike those of 3-methylcholanthrene, also include proliferation of smooth endoplasmic reticulum, stimulation of liver weight gain (Conney, 1967), liver tumour promotion (Schulte-Hermann, 1974), and a general stabilization of liver microsomal protein (Omura, 1979), and are thus pleiotropic. This review primarily

Abbreviations used: PB, phenobarbital; GRE, glucocorticoid response element; TCPOBOP, 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene.

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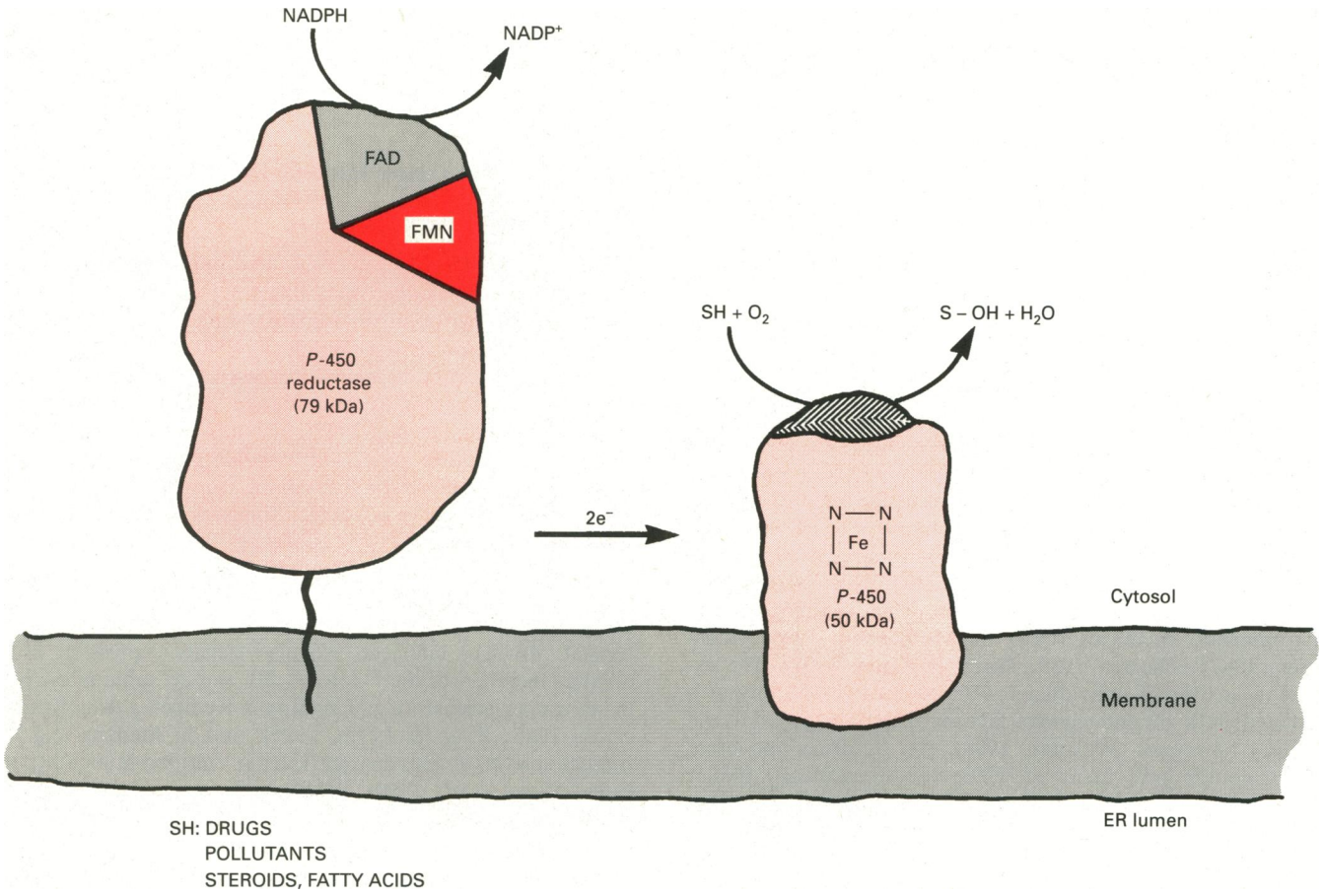

Fig. 1. Hydroxylation of lipophilic substrates (SH) catalyzed by cytochrome *P*-450 enzymes bound to the microsomal membrane

A total of two electrons, derived from NADPH, are transferred from the flavoprotein *P*-450 reductase to the haemoprotein *P*-450. The monooxygenase reaction occurs at the active site of the *P*-450, and involves the incorporation into substrate of one atom of oxygen derived from molecular oxygen (O_2). The active sites of both *P*-450 and its reductase are oriented towards the cytosol. Substrates include drugs, pollutants, steroids and fatty acids. Abbreviation: ER, endoplasmic reticulum.

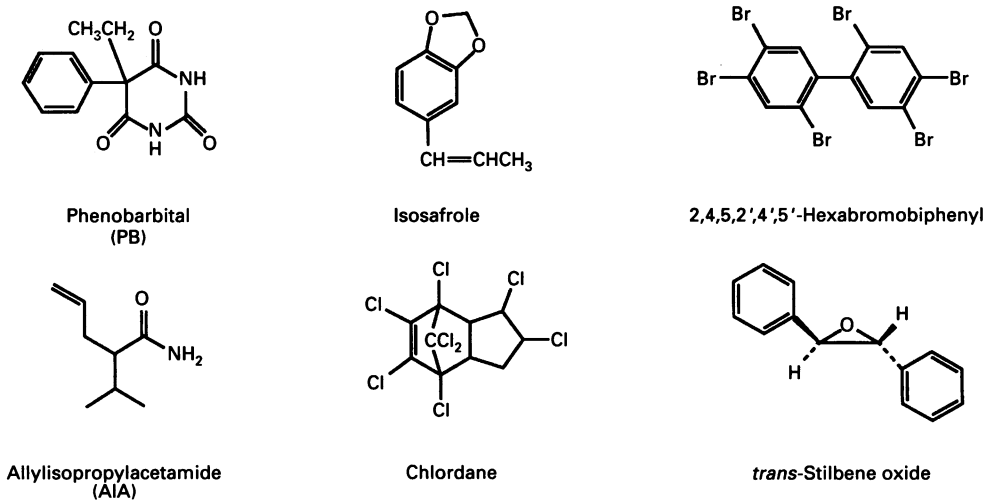

Fig. 2. Structural diversity of PB-like inducers

focuses on the PB induction of rat *P*-450s 2B1 and 2B2, the major PB-inducible *P*-450 enzymes of rat liver; these will be referred to as *P*-450 2B. Selected studies of other PB-inducible enzymes are also discussed. Mechanistic studies of the PB-inducible rabbit and mouse 2B and 2C *P*-450s (e.g. Komori *et al.*, 1988; Zhao *et al.*, 1990; Devore *et al.*, 1985) are not discussed in detail for reason of space limitation. Finally, references are cited in a selective rather than a comprehensive manner, with the intention of providing the reader with useful entry points into the relevant primary literature.

Table 1. PB-inducible rat liver cytochromes *P*-450

Unless indicated otherwise, values refer to increases in liver microsomal *P*-450 protein (Western blot analysis) following treatment of adult male rats with maximally inducing doses of PB, typically 8 mg of PB/100 g body weight by i.p. injection, daily for 4 days. Asterisks indicate induction values determined at the RNA level. See the text for references.

P-450 form	Induction (-fold)[a]	Comments
2A1	2–4	Also inducible by *Ah* receptor ligands (1A1 inducers)
2B1	⩾ 50–100	Major PB-inducible rat *P*-450
2B2	⩾ 20	97% sequence similarity to 2B1, but generally of lower catalytic activity
2C6	2–4	PB induction can be demonstrated in hepatoma cell lines
2C7	20*[a]	Basal level expression increases with puberty, and net PB inducibility drops to ⩽ 2-fold
2C11	2*	Induction of mRNA but not protein
3A1	⩾ 10*	Also inducible by dexamethasone and macrolide antibiotics
3A2	⩾ 10*	Male-specific expression in adult rats

[a] Inducibility determined for immature (3-week-old) rats.

Table 2. PB-inducible enzymes of foreign compound metabolism

With the exception of NADPH:*P*-450 reductase, which is encoded by a single gene that is PB-inducible, the enzyme classes listed are each comprised of multiple enzyme forms (sometimes referred to as isoenzymes). Individual enzyme forms are encoded by separate genes, only some of which are PB-inducible. Individual cytochrome *P*-450 and aldehyde dehydrogenase enzymes can be more highly inducible by PB than are the PB-responsive members of the other enzyme classes.

Cytochrome *P*-450
Aldehyde dehydrogenase
Epoxide hydrolase
NADPH:*P*-450 reductase
UDP-glucuronyltransferase
Glutathione *S*-transferase

2B1 and 2B2 proteins

Cytochromes *P*-450 2B1 and 2B2 are closely related in structure, exhibiting only 14/491 amino acid differences (Suwa *et al.*, 1985). Both *P*-450s contain an *N*-terminal hydrophobic stretch of ~ 20 amino acids that contributes in a major way to membrane anchorage (Monier *et al.*, 1988; Vergeres *et al.*, 1989), a haem-binding peptide that contains a conserved cysteine characteristic of all cytochromes *P*-450, a substrate-binding site, and site(s) that interact with NADPH:*P*-450 reductase but are poorly defined (Nelson & Strobel, 1988; Edwards *et al.*, 1989) (Fig. 3). Given their high degree of amino acid sequence similarity, it is not surprising that 2B1 and 2B2 are immunochemically crossreactive. They can, however, be distinguished on Western blots by virtue of their slightly different migrations on SDS/polyacrylamide gels (Waxman & Walsh, 1982; Ryan *et al.*, 1982). The corresponding mRNAs can be selectively monitored, quantitatively by Northern and slot blotting or by solution hybridization using gene-specific oligonucleotide probes (Omiecinski *et al.*, 1985), or qualitatively using a RNAase protection assay (Friedberg *et al.*, 1990).

P-450 2B1 actively metabolizes a broad spectrum of lipophilic drugs and steroidal substrates, including androgens such as androstenedione, which is subject to a regioselective hydroxylation at the 16β position (Waxman, 1988). Purified *P*-450 2B2 exhibits a similar, but distinguishable, substrate specificity profile, but is several-fold less active than 2B1 with many mono-

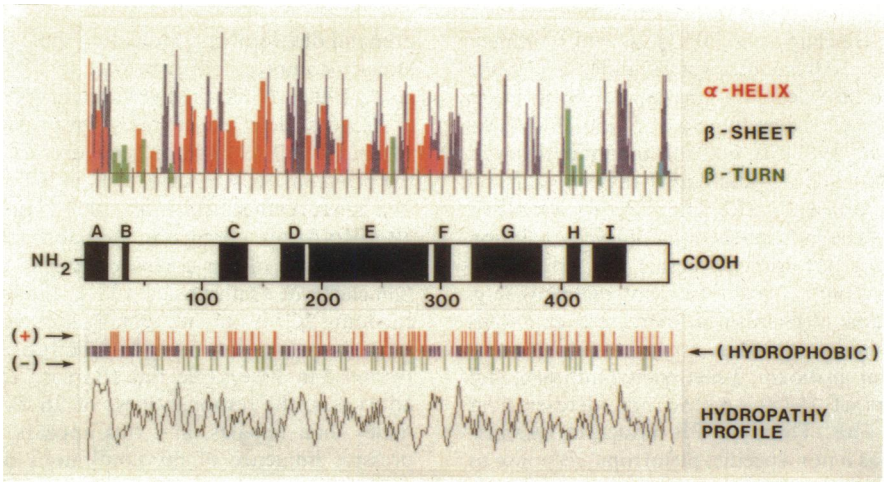

Fig. 3. Proposed functional domains of *P*-450 IIB1 (*P*-450 2B1), based on structural analysis of this and other microsomal *P*-450s and supporting experimental data described in the literature

Shown are: (*a*) predicted occurrences of elements of secondary structure (top panel); (*b*) a linear representation of *P*-450 2B1, with functional domains designated A–I (see below) (second panel); (*c*) distribution of charged and hydrophobic amino acid side chains (positive charges, red; negative charges, green; hydrophobic side chains, purple) (third panel); (*d*) hydropathy profile (bottom panel). Predicted functional domains include: A, membrane insertion region; B, proline-rich segment of unknown function; C, multifunctional region, which includes putative site of interaction with cytochrome b_5, site of phosphorylation by cyclic AMP-dependent protein kinase, and several amino acid residues important for the regioselectivity of steroid hydroxylation; D, putative internal halt-transfer sequence; E, hypervariable region that is probably important for substrate specificity; F, O_2-binding site (Thr-301); G, putative site of interaction with NADPH:*P*-450 reductase; H, conserved segment; I, conserved axial cysteine/haem-binding domain. The figure is based on Holsztynska & Waxman (1989) and was prepared in collaboration with Dr. E. J. Holsztynska.

oxygenase substrates (Waxman & Walsh, 1982; Ryan *et al.*, 1982). 2B1 and 2B2, do, however, appear to exhibit distinct differences in the regioselectivity of 7,12-dimethylbenz[*a*]-anthracene hydroxylation (Christou *et al.*, 1987). In the case of 2B1, major changes in hydroxylation regioselectivity towards steroid substrates can occur by alteration of only two amino acids (Aoyama *et al.*, 1989). The higher catalytic activity of purified 2B1 as compared to purified 2B2 may be an inherent characteristic that relates to its distinct haem environment (Wolf *et al.*, 1988). Alternatively, the lower activity of 2B2 may reflect a susceptibility of this *P*-450 to denaturation during purification, or perhaps a requirement for specific microsomal phospholipids that are absent from the standard reconstituted system (cf. the requirement of phosphatidylserine for reconstitution of 3A *P*-450s in active form; Imaoka *et al.*, 1988; Halvorson *et al.*, 1990). Other data suggest, however, that the catalytic activity of purified 2B2 is similar to that contributed by that *P*-450 form in liver microsomes, and that it is 2B1 which undergoes significant activation in going from the microsomal membrane to a purified, reconstituted system (Christou *et al.*, 1989).

Two-dimensional gel analysis has revealed the existence of several apparent allelic variants of 2B1 and 2B2 (Rampersaud & Walz, 1983), at least some of which are fully active catalytically (Ryan *et al.*, 1982*a*; Oesch *et al.*, 1989). Differences in the PB inducibility of these variants are not apparent. Charge variants of both 2B1 and 2B2 that appear to reflect phosphorylation of the *P*-450 protein can also be observed on such gels, and are probably catalytically inactive (Koch & Waxman, 1989). A variant polypeptide derived from an alternatively spliced 2B2 precursor RNA has also been predicted (Lacroix *et al.*, 1990), but has not been characterized at the protein level.

In uninduced rat liver, 2B2 protein is present at a low, but measurable, level, whereas 2B1 protein expression is at least 5–10-fold lower, and in many investigators' hands is undetectable. Both *P*-450s are elevated significantly above these basal levels (an estimated 20 to > 100-fold increase) upon exposure of rats to PB and PB-like inducers (e.g. Dannan *et al.*, 1983; Christou *et al.*, 1987; Yamazoe *et al.*, 1987). Fasting and exposure to acetone or ethanol will also induce *P*-450s 2B1 and 2B2 (Johansson *et al.*, 1988; Sinclair *et al.*, 1991), as will treatment with the porphyrinogen 2-allylisopropylacetamide, a PB-like inducer (Fig. 2) that can also decrease microsomal haem pools via suicidal inactivation of the haem moiety of *P*-450 2B (Marks *et al.*, 1988; Dwarki *et al.*, 1987). In general, the induced levels of 2B1 protein and mRNA are 2–3-fold higher than the induced 2B2 levels, with the response of 2B1 and 2B2 to 4-*n*-alkyl-methylenedioxybenzene-type inducers being a notable exception (Marcus *et al.*, 1990). It is not known whether the structurally diverse PB-like inducers (Fig. 2) and treatment regimens (e.g. fasting) all act via the same mechanism to increase liver *P*-450s 2B1 and 2B2. *P*-450 2B induction does, however, exhibit some inducer specificity, insofar as dioxin, 3-methylcholanthrene, and other lipophilic inducers of *P*-450 1A1 do not increase liver expression of the 2B *P*-450s. Thus, the PB induction response cannot be characterized as a non-specific, pleiotropic response to lipophilic chemicals.

Tissue specificity and developmental regulation

PB induces the expression of *P*-450s 2B1 and 2B2 in liver to a much greater extent than in extrahepatic tissues. PB can also induce these *P*-450s in the adrenal, although the maximal level of 2B protein that accumulates in that tissue is < 5% of that present in PB-induced liver (Christou *et al.*, 1987). In small intestine, PB induces expression of 2B1 but not 2B2, while in lung and testes 2B1 is apparently expressed constitutively but is not PB-inducible (Omiecinski, 1986; Christou *et al.*, 1987; Traber *et al.*, 1990). Within a single organ, distinct regions or cell types may be differentially responsive to PB. For instance, greater induction of *P*-450s 2B1 and 2B2 occurs in cells in the proximal region as compared to cells in the distal region of the small intestine (Traber *et al.*, 1990). In the case of liver, *in situ* hybridization studies have revealed that PB-inducible 2B1 and 2B2 mRNAs are both distributed uniformly across the centrilobular and mid-zonal regions of the hepatic lobule, while a small band of cells immediately surrounding the periportal tract is refractory to PB induction (Hassett *et al.*, 1989; Traber *et al.*, 1989; Chianale *et al.*, 1986). The zonal distribution of PB-inducible *P*-450 2B probably relates to differences in micro-environment generated by hepatic circulation across the liver lobule, as suggested by the dependence of this distribution on the dose of PB (Bars & Elcombe, 1991). PB-induced expression of liver *P*-450 3A1 mRNA is predominantly centrilobular, a distribution that is clearly distinct from that of the 2B mRNAs (Omiecinski *et al.*, 1990). Distinct mechanisms could thus be responsible for PB induction of 2B as compared to 3A *P*-450s. This suggestion is supported by the following three observations: (1) a lower dose of PB is required for induction of 2B1 and 2B2 mRNAs than for induction of 3A1 mRNA (Kocarek *et al.*, 1990); (2) the 2B and 3A *P*-450s are differentially responsive to cycloheximide (Burger *et al.*, 1990; also see below); (3) structure–activity analysis among a series of polychlorinated biphenyl inducers has revealed different requirements with respect to the number and positions of the chlorines necessary for induction of 2B as compared to 3A *P*-450s (Schuetz *et al.*, 1986). PB induction of *P*-450 3A can be achieved in cultured hepatocytes, indicating that this induction is not an indirect effect resulting from an increased availability of endogenous glucocorticoids in the liver following PB treatment (Schuetz *et al.*, 1986).

P-450s 2B1 and 2B2 are regulated in a similar manner during rat liver development. *P*-450 2B1 and 2B2 mRNA are both expressed at very low constitutive levels in fetal rat liver [requiring polymerase chain reaction (PCR) methods for detection], where they can be transplacentally induced by PB as early as day 15 of gestation (Omiecinski *et al.*, 1990; Giachelli & Omiecinski, 1986). *P*-450 3A1 mRNA is also inducible by PB at this stage of prenatal development, indicating that these fetal hepatocytes are already competent with respect to PB inducibility (Omiecinski *et al.*, 1990). While the liver-specific transcription factor HNF-1 is also first detected at this stage of gestation (Tsutsumi *et al.*, 1989), HNF-1 recognition sequences are apparently absent from the proximal 1650 bases of 5′ flanking region of the rat 2B1 and 2B2 genes (Omiecinski *et al.*, 1990). Higher induced levels of the 2B mRNAs are attained in animals treated with PB postnatally, with 3 weeks of age corresponding to the most responsive age (Giachelli & Omiecinski, 1986). Similarly, while the mRNA encoding 2C7 is only marginally inducible by PB in adult rats, it (or perhaps a closely related allelic variant) exhibits a striking PB response in 3-week-old rats (Barroso *et al.*, 1988) (Table 1). In adult rats, the responsiveness of 2B *P*-450s to PB is greater in males than females, and this appears to result from the suppressive influence of the continuous plasma growth hormone profile associated with female rats, as demonstrated by studies *in vivo* (Yamazoe *et al.*, 1987) and by experiments carried out in cell culture (Waxman *et al.*, 1990; Schuetz *et al.*, 1990). The suppressive effects of thyroid hormone also contribute to the maintenance of 2B at a low level in uninduced rats (Murayama *et al.*, 1991), just as thyroid hormone suppresses the basal expression of the PB-inducible 3A2 mRNA (Waxman *et al.*, 1990*a*; Ram & Waxman, 1991). The PB-inducibility of 2B1 and 2B2 is also inhibited by interleukin 6, a major mediator of hepatic acute phase reaction (Williams *et al.*, 1991). Aging of male rats is associated with a general demasculinization of

hepatic *P*-450 expression (e.g. Kamataki *et al.*, 1985), including a decrease in the PB-responsiveness of the 2B *P*-450s (Horbach *et al.*, 1990).

CYP2B1 and CYP2B2 genes

Rat liver *cy*tochrome *P*-450s 2B1 and 2B2 are respectively encoded by genes designated *CYP2B1* and *CYP2B2*, which are closely linked on rat chromosome 1 (Rampersaud & Walz, 1987). *CYP2B1* has at least six alleles (Rampersaud & Walz, 1983), is 23 kb long and contains nine exons and eight intervening sequences (Suwa *et al.*, 1985). *CYP2B2*, which has a gene structure similar to *CYP2B1* and is found in at least two alleles, has only 40 coding region basepair substitutions relative to *CYP2B1*, and these result in 14 amino acid substitutions in the final peptide product (97% amino acid sequence similarity) (Suwa *et al.*, 1985). The distribution of these substitutions is not random, but is primarily limited to exons 6–9 (Suwa *et al.*, 1985) and probably reflects one or more gene conversion events (Atchison & Adesnik, 1986).

The transcription initiation site of both *CYP2B* genes is putatively ~ 30 bp upstream from the ATG translation initiation site. A modified TATA box, CATAAAA, is found ~ 20 bp further upstream, and beginning at −255 there is an alternating purine/pyrimidine sequence, $(CA)_5$ for *CYP2B1* and $(CA)_{19}$ for *CYP2B2*. This repetitive sequence is also present in a related mouse gene, *Cyp2b-9* (Lakso *et al.*, 1991), and is proposed to form a Z-helical structure that may be of regulatory significance (Suwa *et al.*, 1985). At −81 of *CYP2B2* there is a GCCAAA sequence, which may correspond to a consensus binding site for nuclear factor I and other CCAAT-box-binding transcription factors (CTF) (Santoro *et al.*, 1988); this sequence is preceded by a 13-bp imperfect palindrome from −56 to −68. The overall segment from −56 to −88 is reportedly protected by a rat liver nuclear extract in a DNAase I footprinting assay (Rangarajan & Padmanaban, 1989). The significance of this DNA-binding interaction is unclear, since it was not reported whether the footprint is influenced by the PB induction status of the liver. Promoter mapping experiments designed to identify possible PB-response elements have not yet been described for these (or other) PB-responsive eukaryotic *P*-450 genes (see, however, bacterial *P*-450 gene studies, below). The 5′-flanking sequence of the 2B2 gene has, however, been shown to contain a consensus glucocorticoid response element (GRE) consisting of a pentadecamer around −1357 to −1343 from the transcription start site and a CCAAT sequence approximately 15 bp further upstream that can confer dexamethasone inducibility to heterologous genes (Jaiswal *et al.*, 1987, 1990); together these two elements may comprise a glucocorticoid responsive unit (GRU) (see Grange *et al.*, 1991).

Structure and regulation of other CYP2B genes

Six to eight other genes or pseudogenes homologous to *CYP2B1* and *CYP2B2* have been identified in the rat genome (Atchison & Adesnik, 1983; Mizukami *et al.*, 1983; Giachelli *et al.*, 1989). At least two of these genes are expressed in the liver. One of these encodes *P*-450 2B3 (77% similar to 2B1 and 2B2 at the amino acid level), which is expressed in liver constitutively but is not PB-inducible (Labbe *et al.*, 1988), while the other, *CYP2B8* (trivial designation, gene IV) is expressed at a very low level (detectable only by PCR) that can be increased ~ 6-fold by PB treatment (Giachelli *et al.*, 1989). The 5′ sequence of *CYP2B8* exhibits high (73%) nucleotide similarity to *CYP2B2* from nucleotide −77 to the transcription start site, but this similarity drops to only 36% from nucleotides −311 to −503. A PB-inducible mitochondrial *P*-450 protein that is structurally and immunochemically similar to *P*-450 2B1 has also been described (Shayiq & Avadhani, 1990). This *P*-450, designated *P*-450 mt4, may conceivably be encoded by one of the unidentified rat 2B-related genes (Atchison & Adesnik, 1983; Mizukami *et al.*, 1983).

A human 2B3 *P*-450 that has 76–78% amino acid sequence similarity to rat 2B1 has been described at the cDNA level (*P*-450 2B6; Miles *et al.*, 1988; Yamano *et al.*, 1989), but it is not known whether the expression of this human *P*-450 is inducible by PB. In contrast to rat 2B1, human 2B6 expressed in human hepatoma HepG2 cells is essentially inactive with steroid substrates and exhibits only low benzyloxyresorufin metabolism activity (Waxman *et al.*, 1991). Alternative splicing of transcripts generated from the human 2B6 gene appears to be an important mechanism for generation of mRNAs that cannot encode functional 2B6 proteins (Miles *et al.*, 1989).

TRANSCRIPTIONAL REGULATION OF CYP2B GENE EXPRESSION

Transcriptional activation by PB

The induction of *P*-450 2B by PB is primarily due to new 2B protein synthesis that results from an increase in steady state levels of 2B mRNA (Adesnik *et al.*, 1981; Phillips *et al.*, 1981). Thus, PB induction of these *P*-450s apparently does not involve the mRNA stabilization mechanism associated with induction of *P*-450 1A2 by 3-methylcholanthrene (Kimura *et al.*, 1986; Silver & Krauter, 1988), nor does it involve protein stabilization, which contributes to the induction of 3A *P*-450(s) by macrolide antibiotics (Watkins *et al.*, 1986) and the induction of *P*-450 2E1 by acetone (Song *et al.*, 1989) (Table 3). The PB-induced increase in *P*-450 2B1 and 2B2 mRNA levels is, in turn, primarily due to increased transcription of the corresponding *CYP2B* genes. This transcriptional activation is rapid (detectable within 30–60 min), and can reach a level 20–50-fold higher than the basal transcription rate (Hardwick *et al.*, 1983; Atchison & Adesnik, 1983; Pike *et al.*, 1985). These kinetics of induction are distinct from those that characterize PB induction of the NADPH:*P*-450 reductase and epoxide hydrolase genes, which are also transcriptionally activated by PB (Hardwick *et al.*, 1983); thus, different induction mechanisms probably characterize individual PB-responsive genes. Transcriptional activation of *P*-450 2B expression has also been observed in PB-induced small intestine (Traber *et al.*, 1990). Run-on transcription analyses using genomic subclones extending from exon 2 through exon 9 of the 2B2 gene have further demonstrated that there is nearly equimolar transcription across these segments of the gene; thus transcription initiation and not transcription elongation is likely to be the regulated step (Adesnik & Atchison, 1985). The possible occurrence of a PB-regulated transcription pause or transcription arrest within the first intron of *CYP2B2* (cf. transcription arrest within intron 1 of the *c-fos* gene; Mechti *et al.*, 1991) cannot, however, be ruled out by these data.

An inherent limitation of the nuclear 'run-on' assays used in the above studies to monitor *P*-450 2B gene transcription rates is that they cannot distinguish between the *in vitro* synthesized 2B1 and 2B2 transcripts, since both will hybridize to the filter-bound 2B cDNA probes used in the assay. Consequently, it has not yet been formally established that the 2B1 and 2B2 genes are both transcriptionally activated by PB. However, it seems highly likely that other, subsequent steps that could potentially regulate steady state 2B mRNA levels, including hnRNA processing, RNA transport to the cytoplasm and stability of the cytoplasmic mRNAs, are not of major importance. *P*-450 2B gene amplification and rearrangement have also been eliminated as possible induction mechanisms (Pike *et al.*, 1985). Although quantitative discrepancies between increases in steady-state *P*-450 mRNA

Table 3. Cytochrome *P*-450 drug induction mechanisms

P-450 form	Prototypic inducers	Primary induction mechanism
1A1	Dioxin, 3-methylcholanthrene	Transcriptional activation by ligand-activated *Ah* receptor
1A2	3-Methylcholanthrene	mRNA stabilization
2B1, 2B2	Phenobarbital	Transcriptional activation
2E1	Ethanol, acetone, isoniazid	Protein stabilization
3A1	Dexamethasone	Transcriptional activation; apparently proceeds independently from the classical glucocorticoid receptor pathway
3A1	Triacetyloleandomycin	Protein stabilization
4A1	Clofibrate	Transcriptional activation; may be mediated by peroxisome-proliferator-activated receptor

levels and *P*-450 gene transcription rates observed in some systems might suggest that PB induction involves both transcriptional and post-transcriptional steps (e.g. Hansen & May, 1989), data of this type should be interpreted with caution, as they can result from other factors, such as anti-sense RNA transcription (e.g. Fremeau & Popko, 1990; Haley & Waterfield, 1991), or technical limitations with respect to measurement of true basal transcription rates in the run-on transcription assay.

There is, however, some evidence for PB-dependent *P*-450 induction mechanisms that involve post-transcriptional regulation. For instance, PB stimulates a 3-fold increase in mouse liver *P*-450 2a-5 (P450 coh) protein that is not associated with changes in 2a-5 mRNA levels or gene transcription rate (Aida & Negishi, 1991). PB and other mono-oxygenase inducers can also suppress expression of some constitutively-expressed forms of *P*-450 (e.g. Dannan *et al.*, 1983; Waxman, 1984); however, the mechanisms for these effects are probably complex, as they can involve perturbations of circulating hormones that maintain normal expression of the constitutive hepatic *P*-450s (see Yeowell *et al.*, 1987; LeBlanc & Waxman, 1988). At least some of the suppressive effects of PB on constitutive *P*-450s can be translational or post-translational, as indicated by the finding that PB treatment decreases *P*-450 2C11 protein by 30–40% in adult male rat liver, while increasing 2C11 mRNA in the same liver samples by ~2-fold (Shimada *et al.*, 1989). Conceivably, this effect could result from a competition between the constitutive and the inducible apo-*P*-450s for limiting haem pools, or perhaps competition for available membrane surface in the endoplasmic reticulum.

Protein synthesis requirement

Ongoing protein synthesis may be required for PB induction of *P*-450 2B mRNA in rat liver. This is suggested by experiments using the protein synthesis inhibitor cycloheximide, which blocks the PB-induced increase in 2B mRNA accumulation (Bhat *et al.*, 1987; Chianale *et al.*, 1988; Burger *et al.*, 1990) (Table 4). Nuclear run-on analysis of *P*-450 2B transcription rates further indicates that this effect of cycloheximide is at the level of transcription. However, two important mechanistic questions have not been addressed by these studies. First, it is unclear whether the cycloheximide effect reflects a requirement for *ongoing* protein synthesis, e.g. to maintain sufficient levels of a pre-existing, labile protein (e.g. transcription factor) (cf. a labile factor distinct from the glucocorticoid receptor is required for dexamethasone induction of α_1-acid glycoprotein in rat hepatoma cells; Klein *et al.*, 1988), or whether it indicates a requirement for *new* protein synthesis that is stimulated by PB. In the latter instance, PB induction of the 2B genes would be a secondary response, i.e. one that is dependent on induction of a distinct protein factor whose PB-induced synthesis is blocked by cycloheximide. These possibilities may be distinguished by varying the time of cycloheximide pretreatment, with longer pretreatment times expected to inhibit 2B mRNA accumulation in a more complete manner if the cycloheximide-sensitive protein factor is a pre-existing one, but not if it itself is induced in response to PB treatment (cf. Klein *et al.*, 1987). A second important mechanistic question is whether cycloheximide blocks one or more steps necessary for PB induction of 2B3 mRNA (e.g. through loss of a labile PB-activated transcription factor), or whether it simply interferes with basal transcription of the *CYP2B* genes. Although the apparent decrease in basal levels of 2B-related mRNA following cycloheximide treatment of uninduced rats (Chianale *et al.*, 1988) is consistent with this latter possibility, verification that these basal hybridization signals actually correspond to constitutive 2B1, 2B2 mRNA is required before a definitive resolution of this question can be reached.

In contrast to the suppressive effects of cycloheximide on 2B mRNA expression, cycloheximide *enhances* *P*-450 mRNA accumulation following treatment of rat hepatocytes with PB or any of several other *P*-450 3A inducers (Burger *et al.*, 1990). It is not known whether this synergistic effect of cycloheximide involves 3A gene transcription, or whether it is operative at a post-transcriptional step, e.g. by stabilizing *P*-450 3A mRNA. Cycloheximide itself (in the absence of other inducers) significantly induces 3A1 mRNA in female (but not male) rats (Burger *et al.*, 1990), suggesting that its primary action may be to enhance basal expression of the 3A genes, for instance by blocking accumulation of a labile repressor. A labile repressor has been proposed for the *P*-450 1A1 gene, where a 'superinduction' of *P*-450 gene transcription is observed when hepatoma cells are exposed to dioxin in the presence of cycloheximide. This effect of cycloheximide on *P*-450 1A1 is not observed, however, when monitoring the basal level of *P*-450 1A1 expression, and is also not achieved in variant hepatoma cells defective in nuclear translocation of ligand-activated *Ah* receptor (Israel *et al.*, 1985). The action of the putative repressor thus appears to be at the level of 1A1 transcription induced by a functionally active dioxin–*Ah* receptor complex.

Role of haem

Haem is known to regulate the levels of several haemoproteins and enzymes of haem metabolism (Ades, 1990; Padmanaban *et al.*, 1989; Maines, 1984), and could conceivably play a regulatory role in the expression of one or more cytochrome *P*-450 genes. Nuclear haem pools, in particular, have been hypothesized to play a role in regulating the transcriptional induction of *P*-450s 2B1 and 2B2 by PB. Evidence in favour of this proposal derives from a report that the induction of 2B mRNA and 2B tran-

Table 4. Influence of cycloheximide on drug-induced *P*-450 expression

P-450	Effects of cycloheximide treatment
1A1	Synergistic with dioxin ('superinduction'); requires functional dioxin–*Ah* receptor complex.
2B1/2B2	Blocks PB-induced increase in 2B mRNAs; may interfere with basal 2B expression.
3A	Synergistic with PB ('superinduction'); also induces 3A1 mRNA in female, but not male, rat liver, even in absence of PB
2H1/2H2	Induces 2H mRNA in the absence of other known inducers, but does not have a synergistic effect on PB induction.

scription by either PB or 2-allylisopropylacetamide can be blocked by the haem biosynthesis inhibitors $CoCl_2$ and 3-amino-1,2,4-triazole, albeit at doses that are toxic (e.g. 60 mg $CoCl_2$/kg, corresponding to an LD_{50} dose) (Dwarki *et al.*, 1987). The block in PB-induced *P*-450 2B transcription in the $CoCl_2$- and aminotriazole-treated animals, attributed to depletion of nuclear haem, is reportedly reversed by treatment with haemin *in vivo*; however, this haem reversal is effective over a surprisingly narrow range of doses, 25 or 50 μg haemin/100 g body weight, with no effect obtained at 100 μg haemin/100 g body weight (Dwarki *et al.*, 1987). These same investigators have described similar effects of nuclear haem depletion and haem replacement on the 3-methylcholanthrene-inducible transcription of *P*-450 1A1 (Bhat & Padmanaban, 1988), suggesting that haem may be generally required for drug-inducible liver *P*-450 transcription. However, the correct interpretation of these *in vivo* haem restoration experiments is difficult to establish, since it was not determined in control experiments whether the doses of haem used have a stimulatory effect on PB-activated 2B transcription (i.e. in the absence of $CoCl_2$ treatment). [Others have reported that haem decreases mRNA levels of several PB-inducible mRNAs in rat liver (Srivastava *et al.*, 1990), but those effects occur at very high, non-physiological doses of haem.] It is also unclear why the stimulatory effects of *in vivo* haem treatment (interpreted as 'haem reversal') were only achieved over a very narrow range of haemin doses. Finally, it was also reported that addition of haemin *in vitro* to haem-depleted liver nuclei partially reversed the effects of *in vivo* $CoCl_2$ treatment on 2B transcription, as monitored by the run-on transcription assay (Dwarki *et al.*, 1987). This result implies, however, that haem relieves a block at the level of transcriptional elongation, and not initiation, since isolated nuclei are not known to reinitiate *in vitro* under conditions used for the run-on transcription assays (e.g. Derman *et al.*, 1981). This conclusion is at variance with the *in vivo* haem reversal data described in the same study (Dwarki *et al.*, 1987), which imply an effect of haem at the level of *P*-450 2B transcription initiation.

In other experiments described by the same laboratory, a cloned 5′-DNA segment of the 2B2 gene, extending from −179 to +181 relative to the transcriptional start site, was reported to be sufficient to direct transcription from the 2B2 promoter in freeze-thawed nuclei; moreover, the rate of transcription in this novel *in vitro* transcription system was reportedly enhanced when the nuclei were isolated from PB-induced as compared with uninduced rat liver (Rangarajan & Padmanaban, 1989). Gel retardation and Southwestern blotting further suggested that an 85–90 kDa liver nuclear protein can bind to this DNA, and that the extent of binding may be greater (reflecting either higher binding affinity or greater abundance of the binding protein) in liver nuclear extracts prepared from PB-induced as compared with uninduced rats. Furthermore, DNA-binding activity in the nuclear extract was reportedly blocked by *in vivo* treatment with either cycloheximide or $CoCl_2$, which, as described above, inhibited 2B transcription *in vivo*. *In vitro* addition of 10^{-5} M-haemin to the isolated nuclei reversed the inhibitory effect of $CoCl_2$ treatment on *in vitro* transcription of the 2B2 gene fragment, despite the fact that the same dose of haemin was ineffective at restoring transcription of the endogenous 2B gene measured by a 'run-on' assay in an earlier study by this same group (Dwarki *et al.*, 1987). These experiments have been interpreted in terms of a labile, haem-regulated transcription factor that is either induced or activated by PB (Rangarajan & Padmanaban, 1989; Rao *et al.*, 1990). Haem could conceivably modulate the DNA-binding activity of such a transcription factor by binding to it directly, or by activating it through phosphorylation or other post-translational mechanisms [cf. haem regulation of a protein kinase that inhibits globin mRNA translation; Ochoa & de Haro, 1979]. Precedent for such a model is provided by studies of yeast *HAP1* and *ROX1* gene products, which serve as haem-activated transcription activators and repressors of iso-2-cytochrome *c* and other haem-regulated yeast genes (Pfeifer *et al.*, 1989; Lowry & Zitomer, 1988).

Other experiments, however, are at variance with the PB- and haem-regulated transcription model described above, and suggest that haem status has no major effect on the regulation of *P*-450 2B mRNA. In studies carried out in primary rat hepatocytes cultured under conditions where they are responsive to PB (see below), depletion of haem through treatment with succinylacetone, a more specific inhibitor of haem biosynthesis than $CoCl_2$ or aminotriazole, did not decrease the amount of PB-induced *P*-450 2B protein or mRNA (Sinclair *et al.*, 1990*a*). Similar findings were made by a second group when succinylacetone was used to inhibit haem biosynthesis in intact rats (Srivastava *et al.*, 1989). Moreover, studies carried out in cultured chick hepatocytes *in vivo* revealed that inhibitors of haem synthesis have no effect on the PB inducibility of a chicken *P*-450 mRNA, designated 2H (Hamilton *et al.*, 1988) (also see below). The major conclusion of these studies, that haem is not a crucial regulator of PB-inducible *P*-450 transcription should, however, be viewed with some caution, since it is not certain that effective depletion of relevant haem pools was achieved by succinylacetone treatment. Thus, although extranuclear haem levels were apparently altered under the conditions used in these experiments, as judged by the succinylacetone-dependent decrease in holo-*P*-450 2B protein levels and the associated induction of 5-aminolaevulinate synthase, both of which were reversed by haem replacement (Sinclair *et al.*, 1990*a*), it is still possible that succinylacetone is inefficient in depleting the *nuclear* haem pools that are proposed by Dwarki *et al.* (1987) to regulate transcription of the *P*-450 2B genes. Studies on the effects of $CoCl_2$ treatment

and haem replacement on the PB inducibility of 2B mRNA in the cultured hepatocyte system, including direct measurement of both cytoplasmic and nuclear haem pools, should clarify these points and may lead to more definitive conclusions regarding the proposed regulatory role of haem in the transcriptional activation by PB of *P*-450 gene expression.

Dexamethasone interactions

As noted above, the pentadecamer at −1357 to −1343 of the 2B2 gene resembles a glucocorticoid response element (GRE) and can confer dexamethasone inducibility on a chloramphenicol acetyltransferase (CAT) reporter gene driven by a thymidine kinase promoter (Jaiswal *et al.*, 1990). Dexamethasone displays synergy with PB in inducing *P*-450 2B mRNA in adult rat liver (Rao *et al.*, 1990) and in increasing *P*-450 2B protein and activity in cultured hepatocytes (Waxman *et al.*, 1990). The mechanism for this potentiating effect of dexamethasone is not clear, since dexamethasone itself induces 2B mRNA without increasing transcription of the 2B genes (Simmons *et al.*, 1987; Rao *et al.*, 1990). Moreover, dexamethasone reportedly antagonizes PB-inducible *P*-450 2B gene transcription, perhaps via a process that involves action of the glucocorticoid at a negative glucocorticoid response element (nGRE) (cf. Langer & Ostrowski, 1988) to prevent PB-induced factor binding (Rao *et al.*, 1990). By contrast, in the case of the *P*-450 1A1 gene, which contains a GRE within intron 1 (Mathis *et al.*, 1989), expression in the presence of the inducer 3-methylcholanthrene is synergized by the presence of dexamethasone (Sherratt *et al.*, 1990). Finally, although the putative GRE at about −1350 is not required for PB-induction, it has the potential to confer dexamethasone inducibility, and its relationship to binding and regulation at downstream elements may be important in determining basal and/or induced levels of *P*-450 transcription. It might be useful in this regard to examine other *P*-450 genes that are inducible by both dexamethasone and PB, for instance *P*-450s 3A1 and 3A2 (Gonzalez *et al.*, 1986), although the response of those genes to dexamethasone reportedly does not involve a classical glucocorticoid receptor pathway (Schuetz & Guzelian, 1984).

PB induction in non-mammalian systems

Recent studies suggest that certain aspects of the transcriptional regulation of PB-inducible *P*-450s may be conserved between mammalian and non-mammalian species. In chick hepatocytes *in vivo* and in culture, PB treatment results in a 50-fold induction of two *P*-450 mRNAs, designated 2H1 and 2H2. These mRNAs encode highly homologous proteins (92% amino acid similarity) that share 51–56% amino acid sequence similarity with several PB-inducible and constitutive mammalian *P*-450 2C forms (Hansen & May, 1989; 2H1 and 2H2 designated 'IIF1' and 'IIF2' in that study). Like rat *P*-450 2B1 and 2B2, chick *P*-450s 2H1 and 2H2 are transcriptionally activated in response to PB-like inducing agents (Hansen & May, 1989). However, in contrast to the inhibitory effects of cycloheximide on the PB induction of *P*-450 2B in rat hepatocytes (see above), cycloheximide does not block the drug-induced increases of *P*-450 2H mRNAs in cultured chick hepatocytes (Hamilton *et al.*, 1988) (Table 4). Interestingly, cycloheximide itself (Hamilton *et al.*, 1988), as well as other protein synthesis inhibitors, can greatly increase the amount of *P*-450 2H mRNA in chick hepatocytes at doses required for inhibition of protein synthesis (P. Sinclair, personal communication). These findings suggest that a labile repressor acts on the genes for the chick 2H *P*-450s, and this may be mechanistically equivalent to the positive regulation by cycloheximide of rat *P*-450 3A expression discussed above.

In *Bacillus megaterium*, PB and other lipophilic barbiturates induce the expression of two *P*-450s, *P*-450 BM-3 (gene *CYP102*), a naturally occurring, catalytically self-sufficient cytochrome *P*-450-NADPH:*P*-450 reductase fusion protein of unusually high activity, and *P*-450 BM-1 (gene *CYP106*) (Fulco, 1991). Thus, the responsiveness of *P*-450 genes to PB is not confined to eukaryotes, and may have certain features that are broadly conserved in nature. A barbiturate-responsive regulatory region of the bacterial *P*-450 BM-3 gene has been localized through transformation studies to a DNA segment between 0.8 and 1.1 kb upstream from the transcription start site (Wen *et al.*, 1989). This segment of DNA, designated the R2 region, can also confer a striking decrease to the barbiturate-inducible expression of the chromosomally-encoded *P*-450 BM-3 gene, provided that a second regulatory DNA element (R1, localized to a fragment extending from −0.2 kb to +0.1 kb, relative to the transcription start site) is also present. This decrease can be interpreted in terms of a competition by the exogenous plasmid DNA for *trans*-acting factor(s) that confer barbiturate-responsive positive control to the BM-3 gene (Wen *et al.*, 1989).

Other studies have pointed to an intriguing relationship between the regulatory features that govern barbiturate induction of this bacterial *P*-450 gene and PB induction of the *P*-450 2B genes in rat liver. Comparison of the 5′-flanking DNA sequences of the bacterial *P*-450 genes BM-3 and BM-1 to those of rat *P*-450s 2B1 and 2B2 revealed the occurrence of a 17 bp sequence with high conservation amongst the four genes [9/17 bases identical in all four genes, and 13/17 bases identical in BM3, 2B1 and 2B2; sequences found in the region from −200 to −300 relative to the translation start site for the bacterial genes and from −100 to −119 for the rat genes (He & Fulco, 1991)]. Gel retardation analysis using double-stranded synthetic 17-mer oligonucleotide probes revealed that all four sequences bind to the same protein in soluble extracts of *B. megaterium* grown in the absence of barbiturate. PB treatment of the bacterial cells decreased this binding interaction dramatically, suggesting that the 17-bp sequence corresponds to a barbiturate responsive element that interacts with a PB-regulated repressor protein. Gel retardation analysis of the same 17-mer probes incubated with nuclear extracts prepared from untreated and PB-treated rat liver revealed just the opposite response to PB, i.e. weak binding in the uninduced liver extracts and strong binding (i.e. increased binding affinity or increased abundance of the binding protein) in liver extracts prepared from rats treated with PB (He & Fulco, 1991). Although the functional importance of this DNA segment has yet to be demonstrated, it seems likely that it contributes to PB-dependent *P*-450 gene regulation; negative regulation in the case of the *B. megaterium* genes and positive regulation in the case of the rat genes. Interestingly, *in vitro* treatment of the bacterial or rodent liver extracts with PB mimicked the effects of *in vivo* PB treatment on the gel retardation patterns (He & Fulco, 1991). This implies that (*a*) the effects of PB on the binding protein(s) involved in these interactions do not require new protein synthesis and (*b*) putative PB receptor protein(s) may be present in the nucleus in the case of the rat liver extracts. Further study is needed to confirm the validity and generality of these conclusions, and also may reveal the extent to which other mechanistic aspects of the PB induction process are conserved in prokaryotic and eukaryotic systems.

DEVELOPMENT OF PB-RESPONSIVE PRIMARY HEPATOCYTE CULTURE SYSTEMS

A major impediment to cellular and molecular studies of the PB response derives from the absence of a suitable culture system for *in vitro* studies. Cultured cell lines, including most hepatoma

cells, do not express 2B1 or 2B2, either constitutively or following exposure to PB. Primary hepatocyte cultures, on the other hand, tend to be phenotypically unstable, and undergo significant morphological and biochemical changes, including dedifferentiation and fetalization that is associated with loss of responsiveness to PB. The capacity to synthesize liver-specific proteins, including *P*-450s, is progressively lost in cell cultures, and cytochromes *P*-450 are typically inducible by PB only slightly if at all (Bissell & Guzelian, 1980; Newman & Guzelian, 1982; Paine, 1990). This dedifferentiation of hepatocytes in culture may reflect the loss of liver-specific transcription factors, which are required for the expression of liver-specific genes (Cereghini *et al.*, 1990; Caron, 1990), including cytochromes *P*-450 (Ueno & Gonzalez, 1990).

Empirical studies have led to the identification of culture parameters that can influence the stability of hepatocytes and/or their maintenance of liver-specific functions in culture. The extracellular matrix, culture medium, and presence of added hormones can affect the phenotype of the cultured cells, as can cell density, and cell–cell and cell–matrix interactions (Enat *et al.*, 1984; Ben Ze'ev *et al.*, 1988). Laminin-rich gel matrices that contain type IV collagen and heparin sulphate proteoglycan (Matrigel) have been more effective than simple collagen matrices at maintaining liver-specific functions and cytochromes *P*-450 (Ben Ze'ev *et al.*, 1988; Schuetz *et al.*, 1988; Bissell *et al.*, 1987). Culture media components that have been shown to facilitate retention of liver-specific functions, including *P*-450 maintenance and inducibility, include selenium, nicotinamide, metyrapone, 5-aminolaevulinate and ascorbate, as well as various hormones and growth factors (Enat *et al.*, 1984; Engelmann *et al.*, 1985; Steward *et al.*, 1985; Inouye *et al.*, 1989; Paine, 1990). The absence of cysteine, cystine, growth inhibitors (Enat *et al.*, 1984; Paine *et al.*, 1982), growth hormone, and serum (Waxman *et al.*, 1990; Schuetz *et al.*, 1990) can also be important. In some instances, specific media have been identified that perform better with respect to maintenance of liver-specific phenotype and/or *P*-450 expression. For example, T1 medium was shown to be more effective than modified Weymouth's and much better than Leibovitz-15 in this regard (Turner & Pitot, 1989).

By and large, however, the above findings have not led to dramatic improvements in the responsiveness of 2B or other *P*-450s to PB in cultured hepatocytes. Recently, however, methods have been developed that provide a significantly increased responsiveness of primary rat hepatocytes to PB in cells cultured in serum-free Weymouth's or Williams E medium on plates coated with Matrigel (Schuetz *et al.*, 1988) or collagen (Sinclair *et al.*, 1990), or on plates covalently coated with collagen and maintained in serum-free, modified Chee's medium (Waxman *et al.*, 1990). This latter system has the advantage of providing for long-term viability of the hepatocytes (up to several weeks) and enables the cells to respond to PB by induction of near physiological levels of enzymically active 2B1 and 2B2 protein (Waxman *et al.*, 1990) and 2B mRNA. Although it was initially believed that PB-inducible *P*-450 2B expression in cultured hepatocytes requires the use of Matrigel (Schuetz *et al.*, 1988), this is clearly not the case (Waxman *et al.*, 1990). The use in the original Matrigel experiments (Schuetz *et al.*, 1988) of a modified Weymouth's medium (which is inhibitory with respect to *P*-450 2B induction), and not a specific requirement for Matrigel, appears to account for both the absence of a significant response in that study when using collagen-coated plates, and for the comparatively low level of *P*-450 2B expression achieved when using Matrigel (Sinclair *et al.*, 1990). In any event, these culture systems appear likely to provide a useful opportunity for molecular investigations of the mechanisms of PB induction, particularly if they prove to be compatible with transfection methods that have recently been developed for cultured hepatocytes (Pasco & Fagan, 1989; Ginot *et al.*, 1989; Rippe *et al.*, 1990). When carrying out such mechanistic studies, however, it will be important to verify, wherever possible, that the mechanisms elucidated reflect regulatory pathways operative *in vivo* [cf. transcriptional activation of liver *P*-450 1A1 expression by 3-methylcholanthrene treatment *in vivo* versus occurrence of post-transcriptional induction mechanism(s) in primary hepatocyte cultures; Silver & Krauter, 1988].

MECHANISMS OF PB INDUCTION

Several possible mechanisms whereby PB and 'PB-like' inducers activate transcription of the *P*-450 2B genes can be considered (Figs. 4 and 5).

Receptor-dependent induction mechanisms

PB receptors could, in principle, include plasma membrane proteins that bind PB from the extracellular space in a process that leads to the formation of intracellular second messengers; these would in turn activate 2B gene expression (cf. mechanism of action of polypeptide hormones) (extracellular receptor model; Fig. 4, Scheme 1). However, the lipophilic nature of PB and PB-like inducers suggests as more likely an intracellular receptor-based mechanism, perhaps analogous to that used by steroid hormones. Thus, the binding of PB to an intracellular receptor protein could activate a latent DNA-binding activity of the receptor. This would lead to the binding of activated receptor to regulatory DNA sequences within PB-responsive genes, a step that can be coupled to the transcriptional activation of target gene expression (Fig. 4, Scheme 2). In this simplest of schemes, a single receptor protein would provide both ligand specificity and target gene selectivity for PB induction. Moreover, the activated PB receptor would act as a transcription factor, enabling it to directly transduce its signal to the transcription machinery. This general mechanism is common to a diverse class of endogenous lipophilic hormones and other activators of the steroid/thyroid receptor superfamily transcription factors, including steroid hormones, thyroid hormones, and retinoic acid (Evans, 1988). It is, apparently, also utilized by dioxin and other polycyclics in their interaction with the *Ah* receptor, leading to induction of family 1A *P*-450, (Whitlock, 1986; Nebert *et al.*, 1990; Landers & Bunce, 1991). A receptor-dependent induction mechanism such as this need not be direct; as, for instance, when an inducer–receptor complex activates transcription of one gene, whose product then induces the target gene of interest (cf. discussion of cycloheximide inhibition of new versus ongoing protein synthesis, above). In both the direct and the indirect mechanisms, an activated ligand–receptor complex could serve as a 'competence factor'; that is, a factor necessary but not sufficient for induction of *P*-450 gene expression. Such a mechanism appears to characterize the induction of *P*-450 1A1 by dioxin, as indicated by (*a*) the poor correlation between the tissue specificity of dioxin-inducible *P*-450 1A1 gene expression and the tissue distribution of the *Ah* (i.e. dioxin) receptor (Dunn *et al.*, 1988), and (*b*) the demonstrated requirement for an 87 kDa basic helix–loop–helix protein, encoded by the *arnt* gene, which enables the ligand-binding subunit of the *Ah* receptor to translocate from the cytosol to the nucleus upon binding ligand (Hoffman *et al.*, 1991).

Although models for PB induction involving the binding of PB to an intracellular receptor protein are attractive and may derive precedent from the activation of other xenobiotic-inducible *P*-450 genes, candidate PB receptor proteins have not been identified, despite much effort and biochemical investigation (e.g. Tierney & Bresnick, 1981). These negative results do not, of

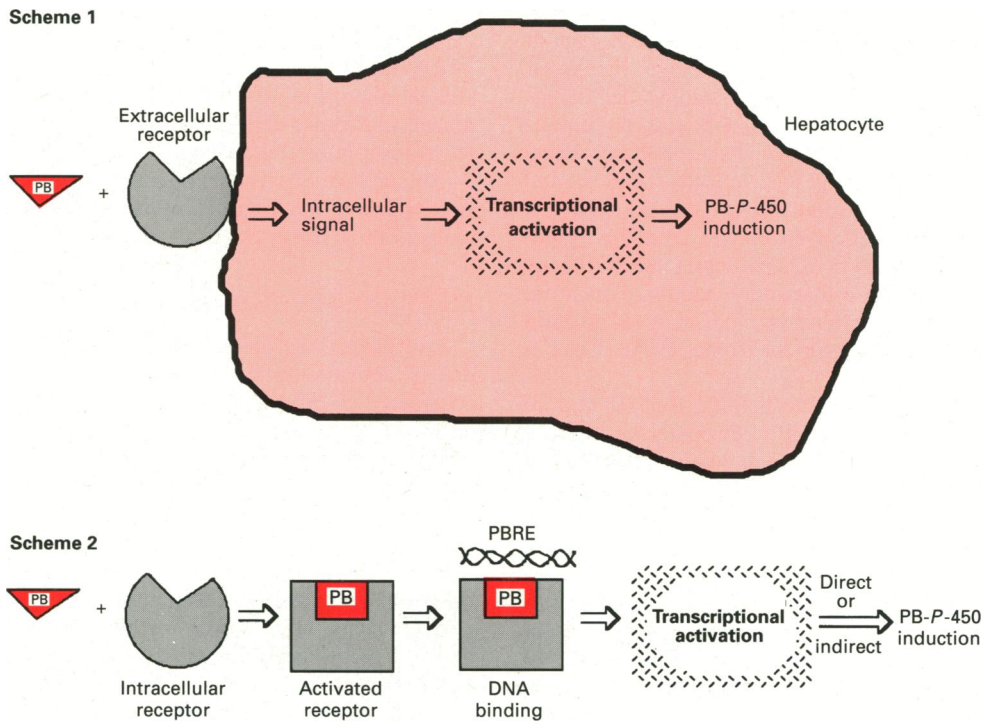

Fig. 4. PB receptor-dependent *P*-450 induction mechanisms

Scheme 2 (intracellular PB receptor) is favoured (see the text) and postulates a conformational change upon binding of PB. The receptor is thereby activated to a form that binds to a PB responsive element (PBRE) in target genes leading to their transcriptional activation.

course, eliminate receptor-dependent mechanisms for PB induction, as only ligand–receptor complexes that are abundant or high affinity are likely to be detected by classical biochemical methods. It may be useful, therefore, to evaluate the applicability of other potentially important characteristics of a PB receptor-mediated induction mechanism. These could include (*a*) the regulation of specific biological processes by PB and PB-like inducers, (*b*) a tissue-specific responsiveness to PB, (*c*) a saturable dose–response curve, (*d*) structure–activity relationships among the various PB-like inducers, (*e*) existence of possible agonists and competitive antagonists of the induction process, and (*f*) genetic differences in response to the inducer. In order to determine whether a putative PB-binding protein in fact functions as a receptor, it may also be important to ascertain whether the ligand-activated protein is present in the nucleus and whether the magnitude of the induction response correlates with the concentration of the inducer-bound protein (Okey, 1990; Poland *et al.*, 1980).

PB induction of 2B *P*-450s does exhibit tissue-specificity (see above) and a saturable dose–response curve both *in vivo* (e.g. Lubet *et al.*, 1985) and in cultured hepatocytes (Kocarek *et al.*, 1990). In contrast, in the case of the PB-inducible bacterial fatty acid mono-oxygenase *P*-450 of *Bacillus megaterium*, induction increases linearly with PB concentration up to the drug's solubility limit (8 mM at 35 °C) (Narhi & Fulco, 1982; also see Wen *et al.*, 1989), a finding that is more suggestive of a receptor-independent mechanism in that system. While it is true that active, PB-like inducers in both eukaryotes (Poland *et al.*, 1980; Hansch *et al.*, 1990) and prokaryotes (Kim & Fulco, 1983) include a huge variety of chemicals with no obvious structural relationships, other than their general lipophilicity, this does not preclude receptor-dependent mechanisms. For instance, the insecticide chlordecone and other chemicals structurally unrelated to oestradiol can bind to the oestrogen receptor to elicit hormone-like effects (Hammond *et al.*, 1979). Moreover, structurally diverse drugs and other chemicals that induce *P*-450 4A gene expression and peroxisome proliferation all appear to activate the peroxisome proliferator receptor, albeit over a wide range of effective doses (Issemann & Green, 1990). These observations are consistent with the hypothesis that PB and PB-like *P*-450 inducers bind to a common receptor with a 'sloppy fit' or an 'elastic recognition site' (Okey, 1990). Unfortunately, PB is only of moderate potency as an inducer [$ED_{50} = 15\ \mu M$ for induction of 2B1 mRNA in cultured rat hepatocytes; Kocarek *et al.*, 1990], and competitive antagonists have not been discovered. 1,4-Bis-[2-(3,5-dichloropyridyloxy)benzene (TCPOBOP), which is a potent inducer of PB-inducible murine *P*-450s, neither induces nor antagonizes PB induction of the *P*-450 genes in intact rats or guinea pigs at the low doses found to be effective in mice (Poland *et al.*, 1981; Raunio *et al.*, 1988). Recent studies have shown, however, that this compound is actually a good inducer of 2B *P*-450 in rat hepatocyte cultures [$ED_{50} \sim 0.7\ \mu M$ (TCPOBOP) versus $\sim 15\ \mu M$ (PB) for induction of *P*-450 2B-dependent benzyloxyresorufin *O*-dealkylase activity; P. Sinclair & W. J. Bement, personal communication]. TCPOBOP could thus serve as a useful probe for studies of the putative PB receptor using this cellular system.

Genetic differences in the responsiveness of 2B *P*-450s to PB have been observed. In the case of the QdJ:SD strain of Sprague–Dawley rat, the PB responsiveness of 2B2 but not 2B1 is reportedly impaired, and the low expression of 2B2 in the affected rats shown to be a recessive trait caused by a single gene mutation (Hashimoto *et al.*, 1988). Analysis of 800 nucleotides of 5′-flanking sequence of the 2B2 gene did not reveal any differences between the wild-type and mutant rat genomic DNA, leading the authors of that study to speculate that the mutation resides in a *trans*-acting factor required for the PB response. However, since even basal 2B2 expression was not detectable, it seems more likely that the mutation in these rats involves a defect in the 2B2 gene (e.g. splicing defect) rather than a defect in its PB in-

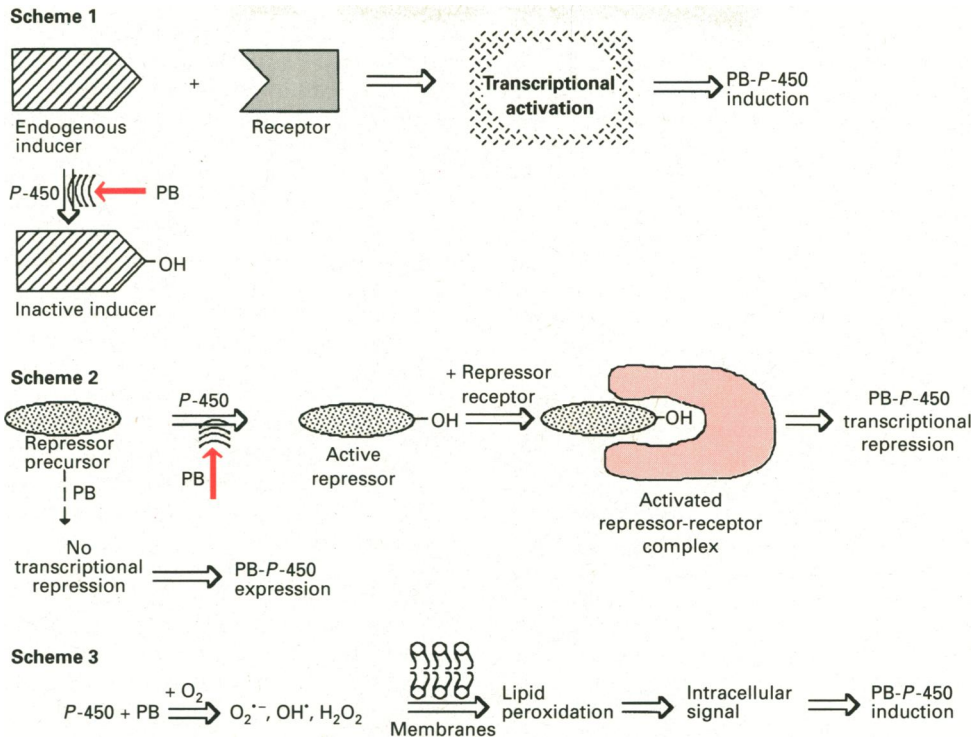

Fig. 5. PB induction mechanisms involving direct interaction of PB with cytochrome *P*-450

PB is postulated to block cytochrome *P*-450-catalyzed metabolism of an endogenous inducer (Scheme 1) or a repressor precursor (Scheme 2) (red arrows). In Scheme 3, direct interactions of PB with cytochrome *P*-450 are proposed to lead to oxygen consumption not coupled to substrate hydroxylation, with the resultant formation of chemically reactive reduced oxygen species, including lipid peroxides. These may, directly or indirectly, provide the intracellular signal for PB-*P*-450 induction.

ducibility. An analogous situation apparently accounts for the absence of both basal and PB-inducible 2B2 gene expression in Marshall 520/N rats (Hassett *et al.*, 1989). Potentially more useful models of genetically impaired PB induction include genetically obese Zucker rats, which are reportedly less responsive to transcriptional activation of 2B genes by PB than their lean Zucker counterparts (Koch *et al.*, 1982; Bandyopadhyay *et al.*, 1991), as well as Long–Evans rats characterized as homozygous non-responsive (*rr*) with respect to PB induction of hepatic aldehyde dehydrogenase (Dunn *et al.*, 1989; Deitrich *et al.*, 1972). This latter instance of PB non-responsiveness is particularly interesting, insofar as PB induction of aldehyde dehydrogenase mRNA is impaired, while PB induction of the mRNAs encoding *P*-450s 2B1, 2B2 and the Ya subunit of glutathione *S*-transferase is apparently unaffected. Moreover, the influence of the *r* and *R* alleles is not limited to aldehyde dehydrogenase, since the *RR* genotype, although normally responsive with respect to PB induction of aldehyde dehydrogenase, 2B1 and glutathione *S*-transferase mRNAs, responds to PB by suppressing the basal expression of 2B2 mRNA (Dunn *et al.*, 1989). These findings raise the exciting possibility that *RR* encodes a receptor or other protein component of the PB response pathway that can confer both positive and negative regulation to PB-responsive genes. Determination of the dominance or recessiveness of the *r* allele with respect to PB induction of 2B2 might be helpful in this regard.

Cytochrome *P*-450-dependent induction mechanisms

In view of the fact that viable candidate PB receptors have not yet been identified, alternative, PB receptor-independent mechanisms may be worth considering (Fig. 5). One key feature that must be considered by any such mechanism is the ability of PB and structurally diverse PB-like inducers to activate expression of essentially the same subset of *P*-450 genes (Table 1). One possible common site of action of these lipophilic chemicals is the substrate-binding site of cytochrome *P*-450 itself (Fonne & Meyer, 1987). For pyrazoles, there is a high correlation between *P*-450 binding, measured in isolated liver microsomes, and induction of *P*-450 activity in cultured hepatocytes (Cornell *et al.*, 1987). Similarly, several *P*-450 2B inducers can bind to 2B or other *P*-450s and give rise to responses other than substrate oxygenation. Binding interactions with non-substrate *P*-450 ligands, such as α-naphthoflavone and metyrapone, can include haem binding associated with spectral changes, *P*-450 inhibition and/or *P*-450 activation (e.g. Murray & Reidy, 1990; Waxman & Walsh, 1983). Thus, one may propose, as a working hypothesis, that *P*-450 2B induction involves, at an early step, interaction of PB with one or more constitutively expressed *P*-450s, or perhaps even with *P*-450 2B2 itself, which is expressed at a low but detectable level in uninduced rat liver (e.g. Christou *et al.*, 1987). According to this model, induction of 2B expression might be mediated by an endogenous substrate of the 2B *P*-450s, perhaps a steroid that is normally present in hepatocytes at a very low level due to its metabolism by the basal *P*-450 2B2. Binding of PB to 2B2 would block metabolism of the endogenous substance, thereby increasing its steady state concentration beyond the threshold required for transcriptional activation of *CYP2B1* and *CYP2B2* (Fig. 5, Scheme 1). An analogous mechanism apparently contributes to the low basal level expression of *P*-450 1A1 in liver and also accounts for the marked elevation of *P*-450 1A1 mRNA in mutant hepatoma cells that contain mis-sense mutations in the 1A1 gene (RayChaudhuri *et al.*, 1990). In an alternative formulation of this hypothesis discussed by Burnet *et al.* (1986), an endogenous steroid (e.g. testosterone) might serve as a 'repressor precursor' that is metabolized to an active repressor (e.g. 16β-hydroxytestosterone, a metabolite selectively formed by 2B

P-450s; Waxman, 1988) by the 2B protein that is expressed constitutively. Binding of PB to the basal, constitutive 2B *P*-450s would inhibit formation of the active repressor, leading to derepression of *CYP2B* transcription (Fig. 5, Scheme 2). Both these models would explain the transcriptional activation of *CYP2B* by large numbers of structurally diverse chemicals as proceeding through one or perhaps a few endogenous compounds, for which one or a few specific receptor proteins would be postulated to exist.

If PB induction proceeds via either the endogenous inducer model or the repressor precursor model, two observations suggest that *P*-450s 2B1 and 2B2 *per se* are probably not directly involved in metabolism of the putative endogenous compound. First, prolonged suppression of the catalytic activities of *P*-450s 2B1 and 2B2 by treatment with the mechanism-based, irreversible *P*-450 inactivator 1-aminobenzotriazole neither induces *P*-450s 2B1 and 2B2, nor blocks their induction by PB (Ortiz de Montellano & Costa, 1986). By contrast, this same inactivator effectively blocks clofibrate induction of both peroxisomal fatty acid β-oxidation and the *P*-450 4A1-catalysed formation of fatty acid metabolites that is postulated to lead to this peroxisome proliferative response (Chan *et al.*, 1991). Second, a sustained, high-level activation of the 2B genes might be difficult to achieve by such a mechanism, since even a moderate increase in 2B, which is detectable at the mRNA level as early as 3 h after PB treatment (Adesnik *et al.*, 1981), might lead to efficient metabolism of the endogenous inducer/repressor precursor, and thereby block further gene activation. An alternative model that would not be subject to these two concerns could involve inactivation of the endogenous inducer or repressor precursor by one or more constitutive *P*-450s (i.e. not 2B1 or 2B2), whose activities could be inhibited or otherwise modulated by their direct interaction with PB or other PB-like 2B inducers.

As a variation on this theme, one might consider as candidate endogenous *P*-450 2B inducers those compounds that are generated as a direct consequence of the interaction of PB with one or more constitutive *P*-450s. For instance, many *P*-450 inducers stimulate NADPH oxidation that is not coupled to substrate oxygenation but is linked to non-productive oxygen activation (e.g. Nordblom & Coon, 1977). This can lead to the formation of superoxide anion, hydroxyl radical and other chemically reactive reduced oxygen species that, in turn, stimulate lipid peroxidation. Conceivably, these peroxides or their secondary metabolites could be involved in the induction process either directly or indirectly (Paine, 1978) (Fig. 5, Scheme 3).

Other receptor-independent induction mechanisms

In addition to the specific effects described above, PB-like inducers might stimulate *P*-450 2B expression by causing general changes in cellular chemistry, such as alterations in membrane fluidity (as caused by anaesthetics) (Ho & Harris, 1981), changes in osmotic pressure (as caused by diuretics) (Poland *et al.*, 1981) or changes in the levels of cell surface receptors or second messengers that mediate the inductive response (e.g. Eckl *et al.*, 1988; Houslay *et al.*, 1981). While some of these changes could be related to the liver hypertrophy induced by PB, others might be central to the mechanism of *P*-450 induction. For instance, chronic PB exposure leads to a major inhibition of phorbol ester-induced translocation of protein kinase C from the cytosol to the plasma membrane (Brockenbrough *et al.*, 1991). This would prevent interaction of the kinase with its membrane-bound protein substrates, several of which participate in signal transduction. PB can also inhibit protein kinase C by displacing the kinase C activator diacylglycerol from its binding site (Chauhan & Brockerhoff, 1987). Accordingly, inhibition of protein kinase C might be an early step in the PB induction process. Indeed, the protein kinase C activator 12-*O*-tetradecanoylphorbol 13-acetate (TPA) can partially block the increase in hepatic microsomal *P*-450 activity in PB-treated hypophysectomized rats (Steele & Virgo, 1988) and can also decrease the *P*-450 2B-associated aldrin epoxidase activity present in several rat hepatoma cell lines (Roesch & Wiebel, 1990). However, these effects on protein kinase C might very well influence the regulation of the 2B *P*-450s or their activity without actually contributing to their induction by PB.

In contrast to these decreases in protein kinase C activity, PB treatment leads to a small, but potentially significant, increase in liver cyclic AMP-dependent protein kinase activity in intact rats as an early response to drug treatment (Byus *et al.*, 1976). Increasing the levels of intracellular cyclic AMP can lead to an increase in total *P*-450 levels in isolated rat hepatocytes (Canepa *et al.*, 1985). It is not clear, however, whether this effect is transcriptional and, indeed, the observed induction may very well be related to post-translational events, such as *P*-450 phosphorylation (Koch & Waxman, 1989; Pyerin & Taniguchi, 1989). Other investigators have observed, however, that *in vivo* treatment with cyclic AMP analogues leads to inhibition of the increase in total *P*-450 observed in PB-treated rats (Hutterer *et al.*, 1975). This apparent discrepancy may be explained by the finding that in isolated hepatocytes cyclic AMP exerts a dual effect, with lower cyclic AMP levels inhibiting *P*-450 activities and higher levels stimulating those activities (Berry & Skett, 1988). Again, it is unclear whether these effects are related to changes in *P*-450 protein levels or to other intracellular changes that influence cellular *P*-450 activities.

FUTURE DIRECTIONS

Search for a PB receptor

Approaches to the critical issue of whether PB induction actually involves a PB receptor protein distinct from cytochrome *P*-450 itself could include one or more of the following: (*a*) further biochemical studies aimed at identification of PB-binding proteins that may participate in the induction process; (*b*) genetic and biochemical analysis of PB-non-responsive rat strains that are potentially defective in a PB-receptor-dependent pathway, and (*c*) evaluation of uncharacterized members of the steroid/thyroid receptor superfamily as possible PB receptor candidates. Finally, characterization of *trans*-acting factors that participate in transcriptional activation of the 2B genes by PB might also lead to identification of PB receptor proteins and also elucidate other mechanistic details of the PB induction response.

(*a*) Biochemical studies. Despite the fact that early attempts to detect hepatic PB receptor proteins were unsuccessful (Tierney & Bresnick, 1981), it may be useful to re-examine this question using improved separation techniques, such as f.p.l.c., which might facilitate the identification of candidate binding proteins of moderate affinity. Buffer, pH, ionic strength, ATP and ligand concentrations could be varied in order to optimize conditions for ligand–receptor binding. Molybdate is known to stabilize a variety of intracellular receptors (e.g. Bresnick *et al.*, 1989), and may be useful for stabilization of the putative PB receptor. High-affinity inducers are more likely to form complexes with longer half-lives. Accordingly, useful ligands could include TCPOBOP for studies of PB-binding proteins in either mouse or rat hepatocyte systems.

Immunochemical approaches might also be useful for PB receptor identification. Antibodies raised to a PB hapten, or preferably against a PB-like ligand with high specificity and affinity for the PB receptor, could be used to immunoprecipitate

ligand–receptor complexes or to visualize such complexes using histochemical methods, either of which may improve the sensitivity of detection above that provided by conventional chromatographic methods. These approaches might also provide useful information on the intracellular localization of any binding sites that may thus be identified.

(*b*) Genetic and biochemical analysis of PB-non-responsive strains of rat. Genetic differences in the responsiveness of *P*-450 1A1 to induction by 3-methylcholanthrene and other polycyclic hydrocarbons have proven quite useful for the identification of the *Ah* receptor and characterization of its role in induction of *P*-450 1A1 and other polycyclic-inducible genes (Nebert, 1986). Genetically-determined strain differences in the PB-inducibility of either *P*-450 or aldehyde dehydrogenase, discussed above, might likewise be exploited to help identify PB receptors or other protein factors key to the PB induction process.

(*c*) Consideration of PB receptor as a member of the steroid/thyroid receptor gene superfamily. Another approach to the search for a PB receptor might be based on the hypothesis that such a receptor belongs to the steroid/thyroid receptor gene superfamily (Evans, 1988; O'Malley, 1990), in which case 'orphan' receptors that have already been cloned might be screened for their activation by PB and PB-like inducers. This could be accomplished by construction of chimaeric receptors comprised of the ligand-binding domain of the unidentified receptor and the *N*-terminal and DNA-binding domain of the oestrogen receptor. The ability of PB to activate an oestrogen receptor target gene (e.g. *Xenopus* vitellogenin 5'-flanking sequences linked to CAT) in a transient, *trans*-activation transfection assay could then be evaluated. This approach has been utilized with striking success for the identification of the clofibrate-activatable peroxisome proliferator receptor (Issemann & Green, 1990).

Molecular analysis of regulatory elements with PB-responsive genes

In order to gain further insight into the molecular basis of PB induction, it will be necessary to elucidate the roles of *cis*-acting DNA elements of the 2B genes and *trans* factors that interact with these regulatory DNA sequences, either directly or via basal or liver-specific components of the transcription apparatus. Studies of this type may be facilitated by the use of PB-responsive hepatocyte culture systems, described above, and could lead to the identification of PB-regulated transcription factors, perhaps including PB receptor proteins that play a role in *P*-450 2B gene activation. These studies may also lead to a determination of whether haem-regulated transcription factors, perhaps analogous to those present in yeast (Pfeifer *et al.*, 1989), play a role in transcription of the cytochrome *P*-450 genes. If these primary hepatocyte transfection studies should prove unsuccessful with the PB-responsive genes, one might need to consider other approaches, such as *in vivo* transfection (e.g. Wu *et al.*, 1989) or expression in transgenic mice. Alternatively, *in vitro* transcription, which has proven useful for characterization of the promoters of other liver-specific genes (e.g. Maire *et al.*, 1989; Tsutsumi *et al.*, 1989), including cytochromes *P*-450 (Yoshioka *et al.*, 1990; Ueno & Gonzalez, 1990).

The 85 kDa protein hypothesized to play a haem-dependent regulatory role in mediating PB-inducible expression of the 2B *P*-450s (Rangarajan & Padmanaban, 1989) should be further characterized and its hypothesized regulation by both haem and PB should be investigated in order to establish or refute its proposed role in the induction of *P*-450 2B gene expression. Similarly, the nuclear 17-mer-binding protein of rat liver, which is apparently much smaller than 85 kDa (He & Fulco, 1991), might be identified by using the rat 2B1 or 2B2 17-mer to screen a rat liver cDNA expression library (cf. Singh *et al.*, 1988). Functional studies could then be carried out to establish its precise role in PB-inducible transcription, and to elucidate the biochemical events that contribute to the apparent increase in binding protein activity following PB stimulation (PB-dependent activation of existing binding protein versus PB-stimulated increase in binding protein synthesis). More extensive gel retardation and DNAase footprinting analyses using extracts prepared from uninduced and PB-induced liver, as well as biochemical fractionation of the extracts, may reveal the existence of other PB-regulated transcription factors, including both activators and repressors that interact with regulatory regions of *CYP2B1* and *CYP2B2* in a specific manner. Studies of the dioxin-inducible *P*-450 1A1 gene have revealed the existence of a complex array of factors that interact with overlapping specificities, and probably in a competing manner, with specific xenobiotic response elements (e.g. Saatcioglu *et al.*, 1990). Complexities of this type can be predicted for *CYP2B* and other PB-inducible genes as well.

Other genes that are induced by PB, such as those coding for aldehyde dehydrogenase (Dunn *et al.*, 1989), NADPH:cytochrome *P*-450 reductase (Porter *et al.*, 1990), and the bacterial cytochrome *P*-450 BM3 (Fulco, 1991) could also be investigated using similar approaches in order to complement molecular studies of the *CYP2B* genes. Also of some usefulness as a model system may be the gene encoding *P*-450 2C6 (*CYP2C6*) (Umeno *et al.*, 1988), whose mRNA can be induced by PB 2–4-fold *in vivo* (Friedberg *et al.*, 1986) and is highly responsive to PB in several differentiated rat hepatoma cell lines (Corcos & Weiss, 1988). Finally, an examination of *P*-450 genes that are homologous to *P*-450s 2B1 and 2B2 but are not PB-inducible, such as rat *P*-450 2B3 (Labbe *et al.*, 1988), may provide further insight into the molecular mechanisms that govern the selectivity of PB induction for individual *P*-450 genes. Together, studies such as these are likely to shed further light on the complex array of regulatory pathways and mechanisms that govern the PB-inducible expression of cytochromes *P*-450 and other enzymes active in hepatic drug and steroid metabolism.

Conclusion

In conclusion, phenobarbital, a prototypic inducer of cytochrome *P*-450 and several other drug-metabolizing liver enzymes, transcriptionally activates the genes encoding two members of the rat *P*-450 2B gene subfamily (*CYP2B*) by mechanisms that are now only beginning to be understood. Several models and working hypotheses for the action of PB on liver *P*-450 gene expression can be considered, including receptor-dependent and receptor-independent induction mechanisms. Recent advances in the molecular characterization of the *P*-450 2B1 and 2B2 genes and their responsiveness to phenobarbital in primary hepatocyte cultures should help elucidate the underlying cellular and molecular mechanisms of phenobarbital induction.

Note added in proof

A PB-responsive enhancer domain has recently been localized by transient transfection of chick embryo primary hepatocytes to a region between −5.9 and −1.1 kb of the chicken *CYP2H1* gene (Hahn *et al.*, 1991).

We thank Drs. Milton Adesnik, Peter Sinclair, and Jacqueline Sinclair for productive discussions on some of the ideas and hypotheses presented in the review. Work carried out in the authors' laboratory was supported in part by grant DK33765 (to D.J.W.) from the National Institutes of Health.

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